# PREVENTION OF Bt RESISTANCE DEVELOPMENT

This invention relates to plant cells and plants, the genomes of which are transformed to contain at least two genes, each coding for a different non-competitively binding Bacillus thuringiensis ("B.thuringiensis" or "Bt") insecticidal crystal protein ("ICP") for a specific target insect species, preferably belonging to the order of Lepidoptera or Coleoptera. Such transformed plants have advantages over plants transformed with a single B. thuringiensis ICP gene, especially with respect to the prevention of resistance development in the target insect species against the at least two B. thuringiensis ICPs, expressed in such plants.

This invention also relates to a process for the production of such transgenic plants, taking into account the competitive and non-competitive binding properties of the at least two B. thuringiensis ICPs in the target insect species' midgut. Simultaneous expression in plants of the at least two genes, each coding for a different non-competitively binding B. thuringiensis ICP in plants, is particularly useful to prevent or delay resistance development of insects against the at least two B. thuringiensis ICPs expressed in the plants.

This invention further relates to a process for the construction of novel plant expression vectors and to the novel plant expression vectors themselves, which contain the at least two B. thuringiensis ICP genes encoding the at least two non-competitively binding B. thuringiensis ICPs. Such vectors allow integration and coordinate expression of the at least two B. thuringiensis ICP genes in plants.

BACKGROUND OF THE INVENTION

Since the development and the widespread use of chemical insecticides, the occurrence of resistant insect strains has been an important problem. Development of insecticide resistance is a phenomenon dependent on biochemical, physiological, genetic and ecological mechanisms. Currently, insect resistance has been reported against all major classes of chemical insecticides including chlorinated hydrocarbons, organophosphates, carbamates, and pyrethroid compounds (Brattsten et al., 1986).

In contrast to the rapid development of insect resistance to synthetic insecticides, development of insect resistance to bacterial insecticides such as B. thuringiensis sprays has evolved slowly despite many years of use (Brattsten et al., 1986). The spore forming gram-positive bacterium B. thuringiensis produces a parasporal crystal which is composed of crystal proteins (ICPs) having insecticidal activity. Important factors decreasing the probability of emergence of resistant insect strains in the field against B. thuringiensis sprays are: firstly the short half-life of B. thuringiensis sprays after foliar application; secondly the fact that commercial B. thuringiensis preparations often consist of a mixture of several insecticidal factors including spores, ICPs and eventually beta-exotoxins (Shields, 1987); and thirdly the transitory nature of plant-pest interactions. Many successful field trials have shown that commercial preparations of a B. thuringiensis containing its spore-crystal complex, effectively control lepidopterous pests in agriculture and forestry (Krieg and Langenbruch, 1981). B. thuringiensis is at present the most widely used pathogen for microbial control of insect pests.

Various laboratory studies, in which selection against B. thuringiensis was applied over several generations of insects, have confirmed that resistance against B. thuringiensis is seldom obtained. However, it should be emphasized that the laboratory conditions represented rather low selection pressure conditions.

For example, Goldman et al. (1986) have applied selection with B. thuringiensis israelensis toxin over 14 generations of Aedes aegypti and found only a marginal decrease in sensitivity. The lack of any observable trend toward decreasing susceptibility in the selected strains may be a reflection of the low selection pressure ($LC_{50}$) carried out over a limited number of generations. However, it should be pointed out that Georghiou et al. (In : Insecticide Resistance in Mosquitoes : Research on new chemicals and techniques for management. In "Mosquito Control Research, Annual Report 1983, University of California.") with Culex quinquefasciatus obtained an 11-fold increase in resistance to B. thuringiensis israelensis after 32 generations at $LC_{95}$ selection presssure.

McGaughey (1985) reported that the grain storage pest Plodia interpunctella developed resistance to the spore-crystal complex of B. thuringiensis; after 15 generations of selection with the Indian meal moth, Plodia interpunctella, using a commercial B. thuringiensis HD-1 preparation ("Dipel", Abbott Laboratories, North Chicago, Illinois 60064, USA), a 100-fold decrease in B. thuringiensis sensitivity was reported. Each of the colonies was cultured for several generations on a diet treated with a constant B. thuringiensis dosage which was expected to produce 70-90% larval mortality. Under these high selection presssure conditions, insect resistance to B.

thuringiensis increased rapidly. More recently, development of resistance against B. thuringiensis is also reported for the almond moth, Cadra cautella (McGaughey and Beeman, 1988). Resistance was stable when selection was discontinued and was inherited as a recessive trait (McGaughey and Beeman, 1988). The mechanism of insect resistance to B. thuringiensis toxins of Plodia interpunctella and Cadra cautella has not been elucidated.

The main cause of B. thuringiensis resistance development in both reported cases involving grain storage was the environmental conditions prevailing during the grain storage. Under the conditions in both cases, the environment was relatively stable, so B. thuringiensis degradation was slow and permitted successive generations of the pest to breed in the continuous presence of the microbial insecticide. The speed at which Plodia developed resistance to B. thuringiensis in one study suggests that it could do so within one single storage season in the bins of treated grain.

Although insect resistance development against B. thuringiensis has mostly been observed in laboratory and pilot scale studies, very recent indications of B. thuringiensis resistance development in Plutella xylostella populations in the (cabbage) field have been reported (Kirsch and Schmutterer, 1988). A number of factors have led to a continuous exposure of P. xylostella to B. thuringiensis in a relatively small geographic area. This and the short generation cycle of P. xylostella have seemingly led to an enormous selection pressure resulting in decreased susceptibility and increased resistance to B. thuringiensis.

A procedure for expressing a B. thuringiensis ICP gene in plants in order to render the plants insect-resistant (European patent publication ("EP") 0193259 [which is incorporated herein by reference]; Vaeck et al., 1987; Barton et al., 1987; Fischhoff et al., 1987) provides an entirely new approach to insect control in agriculture which is at the same time safe, environmentally attractive and cost-effective. An important determinant for the success of this approach will be whether insects will be able to develop resistance to B. thuringiensis ICPs expressed in transgenic plants (Vaeck et al., 1987; Barton et al., 1987; Fischhoff et al., 1987). In contrast with a foliar application, after which B. thuringiensis ICPs are rapidly degraded, the transgenic plants will exert a continuous selection pressure. It is clear from laboratory selection experiments that a continuous selection pressure has led to adaptation to B. thuringiensis and its components in several insect species. In this regard, it should be pointed out that the conditions in the laboratory which resulted in the development of insect-resistance to B. thuringiensis are very similar to the situation with transgenic plants which produce B. thuringiensis ICPs and provide a continuous selection pressure on insect populations feeding on the plants. Mathematical models of selection pressure predict that, if engineered insect-resistant plants become a permanent part of their environment, resistance development in insects will emerge rapidly (Gould, 1988). Thus, the chances for the development of insect resistance to B. thuringiensis in transgenic plants may be considerably increased as compared to the field application of B. thuringiensis sprays. A Heliothis virescens strain has been reported that is 20 times more resistant to B. thuringiensis HD-1 ICP

produced by transgenic Pseudomonas fluorescens and 6 times more resistant to the pure ICP (Stone et al., 1989). Furthermore, the monetary and human costs of resistance are difficult to assess, but loss of pesticide effectiveness invariably entails increased application frequencies and dosages and, finally, more expensive replacement compounds as new pesticides become more difficult to discover and develop.

Therefore, it would be desirable to develop means for delaying or even preventing the evolution of resistance to B. thuringiensis.

B. thuringiensis strains, active against Lepidoptera (Dulmage et al., 1981), Diptera (Goldberg and Margalit, 1977) and Coleoptera (Krieg et al., 1983), have been described. It has become clear that there is a substantial heterogeneity among ICPs from different strains active against Lepidoptera, as well as among ICPs from strains active against Coleoptera (Hofte and Whiteley, 1989). An overview of the different B. thuringiensis ICP genes, that have been characterized, is given in Table 2 (which follows the Examples herein).

Most of the anti-Lepidopteran B. thuringiensis (e.g., Bt3, Bt2, Bt73, Bt14, Bt15, Bt4, Bt18) ICP genes encode 130 to 140 kDa protoxins which dissolve in the alkaline environment of an insect's midgut and are proteolytically activated into an active toxin of 60-65 kDa. These ICPs are related and can be recognized as members of the same family based on sequence homologies. The sequence divergence however is substantial, and the insecticidal spectrum, among the order Lepidoptera, may be substantially different (Höfte et al., 1988).

The P2 toxin gene and the cry B2 gene are different from the above-mentioned genes in that they

do not encode high molecular weight protoxins but rather toxins of around 70 kDa (Donovan et al., 1988 and Widner and Whiteley, 1989, respectively).

It has recently become clear that heterogeneity exists also in the anti-Coleopteran toxin gene family. Whereas several previously reported toxin gene sequences from different B. thuringiensis isolates with anti-Coleopteran activity were identical (EP 0149162 and 0202739), the sequences and structure of bt21 and bt22 are substantially divergent (European patent application ("EPA") 89400428.2).

While the insecticidal spectra of B. thuringiensis ICPs are different, the major pathway of their toxic action is believed to be common. All B. thuringiensis ICPs, for which the mechanism of action has been studied in any detail, interact with the midgut epithelium of sensitive species and cause lysis of the epithelial cells (Knowles and Ellar, 1986) due to the fact that the permeability characteristics of the brush border membrane and the osmotic balance over this membrane are perturbed. In the pathway of toxic action of B. thuringiensis ICPs, the binding of the toxin to receptor sites on the brush border membrane of these cells is an important feature (Hofmann et al., 1988b). The toxin binding sites in the midgut can be regarded as an ICP-receptor since toxin is bound in a saturable way and with high affinity (Hofmann et al., 1988a).

Although this outline of the mode of action of B. thuringiensis ICPs is generally accepted, it remains a matter of discussion what the essential determinant(s) are for the differences in their insecticidal spectra. Haider et al. (1986) emphasize the importance of specific proteases in the insect midgut. Hofmann et al. (1988b) indicate that receptor binding is a prerequisite for toxic activity and describe that

Pieris brassicae has two distinct receptor populations for two toxins. Other authors have suggested that differences in the environment of the midgut (e.g., pH of the midgut) might be crucial.

SUMMARY OF THE INVENTION

In accordance with this invention, a plant is provided having, stably integrated into its genome, at least two B. thuringiensis ICP genes encoding at least two non-competitively binding insecticidal B. thuringiensis ICPs, preferably the active toxins thereof, against a specific target insect, preferably against a Lepidoptera or Coleoptera. Such a plant is characterized by the simultaneous expression of the at least two non-competitively binding B. thuringiensis ICPs.

Also in accordance with this invention, at least two ICP genes, particularly two genes or parts thereof coding for two non-competitively binding anti-Lepidopteran or anti-Coleopteran B. thuringiensis ICPs, are cloned into a plant expression vector. Plant cells transformed with this vector are characterized by the simultaneous expression of the at least two B. thuringiensis ICP genes. The resulting transformed plant cell can be used to produce a transformed plant in which the plant cells: 1. contain the at least two B. thuringiensis ICP genes or parts thereof encoding at least two non-competitively binding anti-Lepidopteran or anti-Coleopteran B. thuringiensis ICPs as a stable insert into their genome; and 2. express the genes simultaneously, thereby conferring on the plant improved resistance to at least one target species of insect, so as to prevent or delay development of resistance to B. thuringiensis of the at least one target species of insect feeding on the transformed plant.

Further in accordance with this invention, plant expression vectors are provided which allow integration and simultaneous expression of at least two B. thuringiensis ICP genes in a plant cell and which comprise one or more chimeric genes, each containing in the same transcriptional unit: a promoter which functions in the plant cell to direct the synthesis of mRNA encoded by one of the ICP genes; one or more different ICP genes, each encoding a non-competitively binding B. thuringiensis ICP; preferably a marker gene; a 3' non-translated DNA sequence which functions in the plant cell for 3' end formation and the addition of polyadenylate nucleotides to the 3'end of the mRNA; and optionally a DNA sequence encoding a protease-sensitive protein part between any two ICP genes.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, "B. thuringiensis ICP" (or "ICP") should be understood as an intact protein or a part thereof which has insecticidal activity and which can be produced in nature by B. thuringiensis. An ICP can be a protoxin, as well as an active toxin or another insecticidal truncated part of a protoxin which need not be crystalline and which need not be a naturally occurring protein. In this regard, an ICP can be a chimaeric toxin encoded by the combination of two variable regions of two different ICP genes as disclosed in EP 0228838.

As used herein, "protoxin" should be understood as the primary translation product of a full-length gene encoding an ICP.

As used herein, "toxin", "toxic core" or "active toxin" should all be understood as a part of a protoxin

which can be obtained by protease (e.g., by trypsin) cleavage and has insecticidal activity.

As used herein, "gene" should be understood as a full-length DNA sequence encoding a protein (e.g., such as is found in nature), as well as a truncated fragment thereof encoding at least the active part (i.e., toxin) of the protein encoded by the full-length DNA sequence, preferably encoding just the active part of the protein encoded by the full-length DNA sequence. A gene can be naturally occurring or synthetic.

As used herein, "truncated B. thuringiensis gene" should be understood as a fragment of a full-length B. thuringiensis gene which still encodes at least the toxic part of the B. thuringiensis ICP, preferentially the toxin.

As used herein, "marker gene" should be understood as a gene encoding a selectable marker (e.g., encoding antibiotic resistance) or a screenable marker (e.g., encoding a gene product which allows the quantitative analysis of transgenic plants).

Two ICPs are said to be "competitively binding ICPs" for a target insect species when one ICP competes for all ICP receptors of the other ICP, which receptors are present in the brush border membrane of the midgut of the target insect species.

Two ICPs are said to be "non-competitively binding ICPs" when, for at least one target insect species, the first ICP has at least one receptor for which the second ICP does not compete and the second ICP has at least one receptor for which the first ICP does not compete, which receptors are present in the brush border membrane of the midgut of the target insect species.

A "receptor" should be understood as a molecule, to which a ligand (here a B. thuringiensis ICP,

preferably a toxin) can bind with high affinity (typically a dissociation constant (Kd) between $10^{-11}$ and $10^{-6}$M) and saturability. A determination of whether two ICPs are competitively or non-competitively binding ICPs can be made by determining whether: 1. a first ICP competes for all of the receptors of a second ICP when all the binding sites of the second ICP with an affinity in the range of about $10^{-11}$ to $10^{-6}$M can be saturated with the first ICP in concentrations of the first ICP of about $10^{-5}$M or less (e.g., down to about $10^{-11}$M); and 2. the second ICP competes for the all of the receptors of the first ICP when all the binding sites of the first ICP with an affinity in the range of about $10^{-11}$ to $10^{-6}$M can be saturated with the second ICP in concentrations of the second ICP of about $10^{-5}$M or less.

General Procedures

This section describes in broad terms general procedures for the evaluation and exploitation of at least two B. thuringiensis ICP genes for prevention of the development, in a target insect, of a resistance to the B. thuringiensis ICPs expressed in transgenic plants of this invention. A non-exhaustive list of consecutive steps in the general procedure follows, after which are described particular Examples that are based on this methodology and that illustrate this invention.

In accordance with this invention, specific B. thuringiensis ICPs can be isolated in a conventional manner from the respective strains such as are listed in Table 2 (which follows the Examples). The ICPs can be used to prepare monoclonal or polyclonal antibodies specific for these ICPs in a conventional manner (Höfte et al., 1988).

The ICP genes can each be isolated from their respective strains in a conventional manner. Preferably, the ICP genes are each identified by: digesting total DNA from their respective strains with suitable restriction enzyme(s); size fractionating the DNA fragments, so produced, into DNA fractions of 5 to 10 Kb; ligating such fractions to suitable cloning vectors (e.g., pEcoR251, deposited at the Deutsche Sammlung von Mikroorganismen und Zellculturen ("DSM"), Braunschweig, Federal Republic of Germany, under accession number no. 4711 on July 13, 1988); transforming E.coli with the cloning vectors; and screening the clones with a suitable DNA probe. The DNA probe can be constructed from a highly conserved region which is commonly present in different B. thuringiensis genes which encode crystal protoxins against Coleoptera or Lepidoptera, such as on the basis of an N-terminal amino acid sequence determined by gas-phase sequencing of the purified proteins (EPA 88402115.5).

Alternatively, the desired fragments, prepared from total DNA of the respective strains, can be ligated in suitable expression vectors ( e.g., a pUC vector (Yanisch-Perron et al., 1985) with the insert under the control of the lac promoter) and transformed in E. coli, and the clones can then be screened by conventional colony immunoprobing methods (French et al., 1986) for expression of the toxins with monoclonal or polyclonal antibodies raised against the toxins produced by the strains.

The isolated B. thuringiensis ICP genes can then be sequenced in a conventional manner using well-known procedures (e.g., Maxam and Gilbert, 1980).

At present, several ICP genes have been cloned from different subspecies of B. thuringiensis (Table 2). The nucleotide sequences from several of these B.

thuringiensis ICP genes have been reported. Whereas several sequences are identical or nearly identical and represent the same gene or slight variants of the same gene, several sequences display substantial heterogeneity and show the existence of different B. thuringiensis ICP gene classes. Several lines of evidence suggest that all these genes specify a family of related insecticidal proteins. Analysis of the distribution of B. thuringiensis ICPs in different B. thuringiensis strains by determining the protein composition of their crystals, by immunodetection using polyclonal antisera or monoclonals against purified crystals, or by using gene-specific probes, shows that subspecies of B. thuringiensis might contain up to three related B. thuringiensis ICP genes belonging to different classes (Kronstad et al., 1983).

To express the isolated and characterized gene in a heterologous host for purification and characterization of the recombinant protein, the preferred organism is Escherichia coli. A number of expression vectors for enhanced expression of heterologous genes in E. coli have been described (e.g., Remaut et al., 1981). Usually the gene is cloned under control of a strong regulatable promoter, such as the lambda pL or pR promoters (e.g., Botterman and Zabeau, 1987), the lac promoter (e.g., Fuller, 1982) or the tac promoter (e.g., De Boer et al., 1983), and provided with suitable translation initiation sites (e.g., Stanssens et al, 1985 and 1987). Gene cassettes of the B. thuringiensis ICP genes can be generated by site-directed mutagenesis, for example according to the procedure described by Stanssens et al. (1985 and 1987). This allows cassettes to be made comprising, for example, a truncated ICP gene fragment encoding the toxic core (i.e., toxin) of an ICP or a hybrid gene

encoding the toxic core and a selectable marker according to the procedures described in EPA 88402241.9.

The cells of an E. coli culture, which has been induced to produce a recombinant ICP, are harvested. The method used to induce the cells to produce the recombinant ICP depends on the choice of the promoter. For example, the lac promoter (Fuller, 1982) is induced by isopropyl-B-D-thiogalacto-pyranoside ("IPTG"); the pL promoter is induced by temperature shock (Bernard et al., 1979). The recombinant ICP is usually deposited in the cells as insoluble inclusions (Hsuing and Becker, 1988). The cells are lysed to liberate the inclusions. The bulk of E. coli proteins is removed in subsequent washing steps. A semi-purified protoxin pellet is obtained, from which the protoxin can be dissolved in alkaline buffer (e.g., $Na_2CO_3$, pH 10). The procedure for the ICP Bt2, which is also applicable to other recombinant toxins, has been described by Höfte et al., 1986.

In accordance with this invention, the binding of various ICPs to ICP receptors on the brush border membrane of the columnar midgut epithelial cells of various insect species has been investigated. The brush border membrane is the primary target of each ICP, and membrane vesicles, preferentially derived from the brush border membrane, can be obtained according to Wolfersberger et al., 1987.

The binding to ICP receptors of one or more ICPs (e.g., ICP A, ICP B, etc.) can be characterized by the following steps (Hofmann et al, 1988b):

1. ICP A is labelled with a suitable marker (usually a radioisotope such as $^{125}I$).
2. Brush border membranes are incubated with a small amount (preferably less than $10^{-10}$ M) of labelled

ICP A together with different concentrations of non-labelled ICP A (preferably from less than $10^{-11}$ to $10^{-5}$ M).

3. For all concentrations tested the amount of labelled ICP A bound to the brush border membranes is measured.
4. Mathematical analysis of these data allows one to calculate various characteristics of the ICP receptor such as the magnitude of the population of binding sites (Scatchard, 1949).
5. Competition by other toxins (e.g. ICP B) is preferably studied by incubating the same amount of labelled ICP A with brush border membranes in combination with different amounts of ICP B (preferentially from $10^{-11}$ to $10^{-6}$ M; and subsequently, steps 3 and 4 are repeated.

By this procedure, it has been found, for example, that Bt3 toxin, Bt2 toxin and Bt73 toxin are competitively binding anti-Lepidopteran ICPs for Manduca sexta and Heliothis virescens (See example 6 which follows). Various other combinations of toxins have been found to be non-competitively binding anti-Lepidopteran or anti-Coleopteran toxins (example 6).

Although the concept of competitivity versus non-competitivity of ICP binding does not have any practical importance by itself, the observation of the non-competitivity of two B. thuringiensis ICPs, active against the same target insect, can be put to very significant practical use. This is because a combination of two non-competitively binding B. thuringiensis ICPs can be used to prevent development, by a target insect, of resistance against such B. thuringienis ICPs.

A selection experiment with M. sexta, using Bt2 toxin, Bt18 toxin, and a mixture of Bt2 and Bt18

toxins, has shown that Bt2 and Bt18 are two non-competitively binding anti-Lepidopteran toxins. After 20 generations of selection, a very pronounced reduction in ICP sensitivity was observed in the selection experiments with Bt2 or Bt18 alone (>100 times). The reduction in sensitivity in the selection experiment with a Bt2-Bt18 mixture was only marginal (3 times). This demonstrates the unexpected practical advantage of a simultaneous use of two non-competitively binding ICPs in a situation which models the high selection pressure which will exist with the use of transgenic plants transformed with ICP genes. In this regard, the two resistant strains showed a specific loss in receptor sites for either the Bt2 or Bt18 toxin. In each case, receptor sites for the toxin, which was not used for selection, were not affected or their concentration even increased. Thus, the Bt2 selected strain retained its Bt18 receptors, and the Bt18 selected strain developed an increased number of Bt2 receptors. Indeed, the Bt18 selected strain showed an increased sensitivity for Bt2 along with its increased Bt2 receptor concentration. No significant changes in receptor sites were found in the strain selected against the combined toxins. These findings are described in detail in Example 7 which follows.

A similar mechanism of resistance to Bt has been observed with respect to a strain of diamondback moth, Plutella xylostella. This strain had developed resistance in the field to Dipel which is a commercial formulation of the Bt HD-1 strain. Crystals of Dipel comprise a mixture of several BtICPs, similar to the Bt2, Bt3 and Bt73 proteins which are competitively-binding ICPs. As shown by both insect bioassays and competitive binding studies using Bt2 and Bt15, the Dipel-resistant diamondback moth strain is resistant to

Bt2 protoxin and toxin but maintains full sensitivity to Bt15 protoxin and toxin. This finding is relevant to other combinations of non-competitively binding anti-Lepidopteran or Coleopteran ICPs which are expected to have the same beneficial effect against their common target insects.

Hence, a combination of non-competitively binding ICPs, when directly expressed in a transgenic plant, offers the substantial advantage of reducing the chances of development of insect resistance against the ICPs expressed in the plant. There may be additional benefits because the combined spectrum of two toxins may be broader than the spectrum of a single ICP expressed in a plant (See Examples 8, 9 and 10 which follow).

If, among two competitively binding ICPs, one has a larger binding site population than the other against a given target insect, it will be most advantageous to use the one with the larger population of binding sites to control the target pest in combination with the most suitable non-competitively binding B. thuringiensis ICP. For example, as seen from Example 6, it is preferred to use Bt73 against Heliothis virescens, rather than Bt2 or Bt3, and it is preferred to use Bt3 against Manduca sexta rather than Bt2 or Bt73. The selected gene can then be combined with the best suitable non-competitively binding ICP.

Previously, plant transformations involved the introduction of a marker gene together with a single ICP gene, within the same plasmid, in the plant genome (e.g., Vaeck et al., 1987; Fischoff et al., 1987). Such chimeric ICP genes usually comprised either all or part of an ICP gene, preferably a truncated ICP gene fragment encoding the toxic core, fused to a selectable marker gene, such as the neo gene coding for neomycin

phosphotransferase. The chimeric ICP gene was placed between the T-DNA border repeats for Agrobacterium Ti-plasmid mediated transformation (EP 0193259).

This invention involves the combined expression of two or even more B. thuringiensis ICP genes in transgenic plants. The insecticidally effective B. thuringiensis ICP genes, encoding two non-competitively binding ICPs for a target insect species, preferably encoding the respective truncated ICP genes, are inserted in a plant cell genome, preferably in its nuclear genome, so that the inserted genes are downstream of, and under the control of, a promoter which can direct the expression of the genes in the plant cell. This is preferably accomplished by inserting, in the plant cell genome, one or more chimaeric genes, each containing in the same transcriptional unit: at least one ICP gene; preferably a marker gene; and optionally a DNA sequence encoding a protease (e.g., trypsin)-sensitive or -cleavable protein part intercalated in frame between any two ICP genes in the chimaeric gene. Each chimaeric gene also contains at least one promoter which can direct expression of its ICP gene in the plant cell.

The selection of suitable promoters for the chimaeric genes of this invention is not critical. Preferred promoters for such chimaeric genes include: the strong constitutive 35S promoter obtained from the cauliflower mosaic virus, isolates CM 1841 (Gardner et al., 1981), CabbB-S (Franck et al., 1980) and CabbB-JI (Hull and Howell, 1987); the promoter of the nopaline synthetase gene ("PNOS") of the Ti-plasmid (Herrera-Estrella, 1983); the promoter of the octopine synthase gene ("POCS" [De Greve et al., 1982]); and the wound-inducible TR1' promoter and the TR2' promoter which drive the expression of the 1' and 2' genes,

respectively, of the T-DNA (Velten et al., 1984). Alternatively, a promoter can be utilized which is specific for one or more tissues or organs of the plant, whereby the inserted genes are expressed only in cells of the specific tissue(s) or organ(s). Examples of such promoters are a stem-specific promoter such as the AdoMet-synthetase promoter (Peleman et al., 1989), a tuber-specific promoter (Rocha-Sosa et al., 1989), and a seed-specific promoter such as the 2S promoter (Krebbers et al., 1988). The ICP genes could also be selectively expressed in the leaves of a plant (e.g., potato) by placing the genes under the control of a light-inducible promoter such as the promoter of the ribulose-1,5-bisphosphate carboxylase small subunit gene of the plant itself or of another plant such as pea as disclosed in EP 0193259. Another alternative is to use a promoter whose expression is inducible (e.g., by temperature or chemical factors).

A 3' non-translated DNA sequence, which functions in plant cells for 3' end formation and the polyadenylation of the 3' end of the mRNA sequence encoded by the at least one ICP gene in the plant cell, also forms part of each such chimeric gene. The selection of a suitable 3' non-translated DNA sequence is not critical. Examples are the 3' untranslated end of the octopine synthase gene, the nopaline synthase gene or the T-DNA gene 7 (Velten and Schell, 1985).

The selection of marker genes for the chimaeric genes of this invention also is not critical, and any conventional DNA sequence can be used which encodes a protein or polypeptide which renders plant cells, expressing the DNA sequence, readily distinguishable from plant cells not expressing the DNA sequence (EP 0344029). The marker gene can be under the control of its own promoter and have its own 3' non-translated DNA

sequence as disclosed above, provided the marker gene is in the same genetic locus as the ICP gene(s) which it identifies. The marker gene can be, for example: a herbicide resistance gene such as the sfr or sfrv genes (EPA 87400141); a gene encoding a modified target enzyme for a herbicide having a lower affinity for the herbicide than the natural (non-modified) target enzyme, such as a modified 5-EPSP as a target for glyphosate (U.S. patent 4,535,060; EP 0218571) or a modified glutamine synthetase as a target for a glutamine synthetase inhibitor (EP 0240972); or an antibiotic resistance gene, such as a neo gene (PCT publication WO 84/02913; EP 0193259).

Using A. tumefaciens Ti vector-mediated plant transformation methodology, all chimeric genes of this invention can be inserted into plant cell genomes after the chimaeric genes have been placed between the T-DNA border repeats of suitable disarmed Ti-plasmid vectors (Deblaere et al., 1988). This transformation can be carried out in a conventional manner, for example as described in EP 0116718, PCT publication WO 84/02913 and EPA 87400544.0. The chimeric genes can also be in non-specific plasmid vectors which can be used for direct gene transfer (e.g., as described by Pazkowski et al., 1984; De La Pena et al., 1986). Different conventional procedures can be followed to obtain a combined expression of two B.thuringiensis ICP genes in transgenic plants as summarized below.

I Chimeric gene constructs whereby two or more ICP genes and a marker gene are transferred to the plant genome as a single piece of DNA and lead to the insertion in a single locus in the genome

Ia The genes can be engineered in different transcriptional units each under control of a distinct promoter

To express two or more ICP genes and a marker gene as separate transcriptional units, several promoter fragments directing expression in plant cells can be used as described above. All combinations of the promoters mentioned above in the chimaeric constructs for one ICP gene are possible. Examples of such individual chimeric constructs are described for the bt2 gene in EP 0193259, for the bt13 gene in EPA 88402115.5 and for the bt18 gene in EPA 88402241.9. The ICP gene in each chimeric gene of this invention can be the intact ICP gene or preferably an insecticidally-effective part of the intact ICP gene, especially a truncated gene fragment encoding the toxic core of the ICP. The individual chimeric genes are cloned in the same plasmid vector according to standard procedures (e.g., EP 0193259).

Ib Two genes (e.g., either an ICP and a marker gene or two ICP genes) or more can be combined in the same transcriptional unit

To express two or more ICP genes in the same transcriptional unit, the following cases can be distinguished:

In a first case, hybrid genes in which the coding region of one gene is in frame fused with the coding region of another gene can be placed under the control of a single promoter. Fusions can be made between either an ICP and a marker gene or between two ICP genes. An example of an ICP gene-marker gene fusion has been described in EP 0193259 (i.e., a hybrid truncated bt2-neo gene encoding a Bt2 toxin-NPTII fusion protein).

Another possibility is the fusion of two ICP genes. Between each gene encoding an ICP which still is insecticidally active (i.e., a toxic part of the protoxin), a gene fragment encoding a protease (e.g.,

trypsin) - sensitive protein part should be included, such as a gene fragment encoding a part of the N-terminal or C-terminal amino acid sequence of one of the ICPs which is removed or cleaved upon activation by the midgut enzymes of the target insect species.

In a second case, the coding regions of the two respective ICP genes can be combined in dicistronic units placed under the control of a promoter. The coding regions of the two ICP genes are placed after each other with an intergenic sequence of defined length. A single messenger RNA molecule is generated, leading to the translation into two separate gene products. Based on a modified scanning model (Kozak, 1987), the concept of reinitiation of translation has been accepted provided that a termination codon in frame with the upstream ATG precedes the downstream ATG. Experimental data also demonstrated that the plant translational machinery is able to synthesize several polypeptides from a polycistronic mRNA (Angenon et al., 1989).

II Chimeric constructs with one or more ICP genes that are transferred to the genome of a plant already transformed with a one or more ICP genes

Several genes can be introduced into a plant cell during sequential transformation steps (retransformation), provided that an alternative system to select transformants is available for the second round of transformation. This retransformation leads to the combined expression of ICP genes which are introduced at multiple loci in the genome. Preferably, two different selectable marker genes are used in the two consecutive transformation steps. The first marker is used for selection of transformed cells in the first transformation, while the second marker is used for selection of transformants in the second round of

transformation. Sequential transformation steps using kanamycin and hygromycin have been described, for example by Sandler et al. (1988) and Delauney et al. (1988).

III Chimeric constructs with one or more ICP genes, that are separately transferred to the nuclear genome of separate plants in independent transformation events and are subsequently combined in a single plant genome through crosses.

The first plant should be a plant transformed with a first ICP gene or an F1 plant derived herefrom through selfing (preferably an F1 plant which is homozygous for the ICP gene). The second plant should be a plant transformed with a second ICP gene or an F1 plant derived herefrom through selfing (preferably an F1 plant which is homozygous for the second ICP gene). Selection methods can be applied to the plants obtained from this cross in order to select those plants having the two ICP genes present in their genome (e.g., Southern blotting) and expressing the two ICPs (e.g., separate ELISA detection of the immunologically different ICPs). This is a useful strategy to produce hybrid varieties from two parental lines, each transformed with a different ICP gene, as well as to produce inbred lines containing two different ICP genes through crossing of two independent transformants (or their F1 selfed offspring) from the same inbred line.

IV Chimeric constructs with one or more ICP genes separately transferred to the genome of a single plant in the same transformation experiment leading to the insertion of the respective chimeric genes at multiple loci.

Cotransformation involves the simultaneous transformation of a plant with two different expression vectors, one containing a first ICP gene, the second

containing a second ICP gene. Along with each ICP gene, a different marker gene can be used, and selection can be made with the two markers simultaneously. Alternatively, a single marker can be used, and a sufficiently large number of selected plants can be screened in order to find those plants having the two ICP genes (e.g., by Southern blotting) and expressing the two proteins (e.g., by means of ELISA). Cotransformation with more than one T-DNA can be accomplished by using simultaneously two different strains of Agrobacterium, each with a different Ti-plasmid (Depicker et al., 1985) or with one strain of Agrobacterium containing two T-DNAs on separate plasmids (de Framond et al., 1986). Direct gene transfer, using a mixture of two plasmids, can also be employed to cotransform plant cells with a selectable and a non-selectable gene (Schocher et al., 1986).

The transgenic plant obtained can be used in further plant breeding schemes. The transformed plant can be selfed to obtain a plant which is homozygous for the inserted genes. If the plant is an inbred line, this homozygous plant can be used to produce seeds directly or as a parental line for a hybrid variety. The gene can also be crossed into open pollinated populations or other inbred lines of the same plant using conventional plant breeding approaches.

Of course other plant transformation methods can be used and are within the scope of the invention as long as they result is a plant which expresses two or more non-competitively binding ICPs. In this regard, this invention is not limited to the use of Agrobacterium Ti-plasmids for transforming plant cells with genes encoding non-competitively binding ICPs. Other known methods for plant cell transformations, such as electroporation or by the use of a vector

system based on plant viruses or pollen, can be used for transforming monocotyledonous and dicotyledonous plants in order to obtain plants which express two non-competitively binding ICPs. Furthermore, DNA sequences encoding two non-competitively binding ICPs other than those disclosed herein can be used for transforming plants. Also, each of the ICP genes, described herein, can be encoded by equivalent DNA sequences, taking into consideration the degeneracy of the genetic code. Also, equivalent ICPs with only a few amino acids changed, such as would be obtained through mutations in the ICP gene, can also be used, provided they encode a protein with essentially the same characteristics (e.g., insecticidal activity and receptor binding).

The following Examples illustrate the invention. Those skilled in the art will, however, recognize that other combinations of two or more non-competitively binding B. thuringiensis ICP genes can be used to transform plants in accordance with this invention in order to prevent the development, in a target insect, of resistance to B. thuringiensis ICPs expressed in the transformed plants. Unless otherwise indicated, all procedures for making and manipulating DNA were carried out by the standardized procedures described in Maniatis et al, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory (1982).

EXAMPLE 1: Collection of genes

The collection of anti-Lepidopteran and anti-Coleopteran Bt genes encoding ICPs, which are the subject of the Examples, is described in Table 2 (following the Examples). References for the respective genes are indicated in Table 2. The origin, the isolation and characterization of the Bt genes, which have not been published, are described below. Bt

strains, such as strains HD-1, HD-68, HD-110, and HD-73, are publicly available from the Agricultural Research Culture Collection, Northern Regional Research Laboratory, U.S. Dept. of Agriculture, Peoria, Illinois 61604, U.S.A.

bt3

gene: From B. thuringiensis var. kurstaki HD-1, the ICP was cloned. Characterization of this gene revealed an open reading frame of 3528 bp which encodes a protoxin of 133 kDa. This gene was identical to the one described by Schnepf et al. (1985).

bt73

gene: From B. thuringiensis var HD-73. The ICP gene was cloned as described by Adang et al. (1985).

bt4

gene: A genomic library was prepared from total DNA of strain B. thuringiensis aizawai HD-68. Using the 1.1 kb internal HindIII fragment of the bt2 gene as a probe, a gene designated bt4 was isolated. Characterization of this gene revealed an open reading frame of 3495 bp which encodes a protoxin of 132 kDa and a trypsin activated toxin fragment of 60 kDa. This (insect controlling protein) gene differs from previously identified genes and was also found in several other strains of subspecies aizawai and entomocidus including HD-110. Fig. 13 shows the nucleotide sequence and deduced amino acid sequence of the open reading frame ("ORF") of the bt4 gene extending from nucleotide 264 to nucleotide 3761.

bt14 and bt15

genes: A genomic library was prepared from total DNA of strain B. thuringiensis var. entomocidus HD-110 by partial Sau3A digest of the total DNA and cloning in the vector pEcoR251 (deposited at DSM under

accession number 4711). Using monoclonal antibodies (Höfte et al., 1988), at least three structurally distinct ICPs were identified in crystals of B. thuringiensis entomocidus HD-110. These monoclonal antibodies were used to clone the three different ICP genes from this B. thuringiensis strain. One of these genes is the bt4 gene as described above.

The second gene was called "bt15". Fig. 14 shows the nucleotide sequence and deduced amino acid sequence of the ORF of the bt15 gene, isolated from HD-110, extending from nucleotide 234 to nucleotide 3803. The Shine and Dalgarno sequence, preceding the initiation codon is underlined. This gene has an open reading frame of 3567 bp which encodes a protoxin of 135 kDa and a 63 kDa toxin fragment. A similar gene has been described by Honee et al. 1988, isolated from B. thuringiensis entomocidus 60.5. The bt15 gene differs from the published sequence at three positions: an Ala codon (GCA) is present instead of an Arg codon (CGA) at position 925 and a consecution of a Thr-His codon (ACGCAT) is present instead of a Thr-Asp codon (ACCGAT) at position 1400. (The numbers of the positions are according to Honnee et al., 1988). Another similar gene has been described in EP 0295156, isolated from B. thuringiensis aizawai 7-29 and entomocidus 6-01. The bt15 gene is different from this published nucleotide sequence at three different places : 1) a Glu codon (GAA) instead of an Ala codon (GCA) at position 700; 2) the sequence TGG, CCA, GCG, CCA instead of TGC, CAG, CGC, CAC, CAT at position 1456 and 3) an Arg codon (CGT) instead of an Ala codon (GCG) at

position 2654. (The numbers of the positions are according to EP 0295156).

The third gene isolated was called "bt14". It has an open reading frame of 3621 bp which encodes a 137 kDa protoxin and a 66 kDa activated toxin fragment. A similar gene has been cloned from B.thuringiensis HD-2 (Brizzard and Whiteley, 1988). The bt14 gene differs from the published nucleotide sequence by two nucleotide substitutions: a T instead of a C at position 126, and a C instead of a T at position 448 (the numbers of the positions are according to Brizzard and Whiteley, 1988). In the first case, the Ile codon (ATT or ATC) is conserved whereas in the second case the Tyr codon (TAT) is converted to a His codon (CAC).

bt2

gene: The bt2 gene was cloned as described in EP 0193259.

bt18

gene: Cloning of the bt18 gene was performed as described in EPA 88402241.9.

bt13

gene: The bt13 gene was cloned as described in EPA 88402115.5.

bt21 and bt22

genes: These genes, encoding Coleopteran-active ICPs, were cloned as described in EPA 89400428.2.

EXAMPLE 2 : Construction of gene cassettes and expression of Bt genes in E.coli

1) bt2, bt18: the construction of bt2 and bt18 gene cassettes has been previously described in EPA 86300291.1 and 88402241.9, respectively. Basically, they comprise a truncated gene encoding the toxic core and a hybrid gene comprising the

truncated gene fused in frame to the N-terminus of the neo gene. The gene cassettes are used to transform E. coli to express the Bt2 and Bt18 ICP toxins.

2) bt14, bt15: as described in EPA 88402241.9, gene cassettes for the bt14 and bt15 genes were constructed in order to express the genes in E.coli and in plants.

First, a NcoI site was introduced at the N-terminus of the genes by site-directed mutagenesis.

In the case of the bt15 gene, the conversion of the TT nucleotides, immediately in front of the ATG codon, into CC yielded a NcoI site overlapping with the ATG initiation codon. This site was introduced using the pMa/c vectors for site-directed mutagenesis (Stanssens et al., 1987) and a 28-mer oligonucleotide with the following sequence:

5'-CGGAGGTATTCCATGGAGGAAAATAATC-3'.

This yielded the plasmid pVE29 carrying the N-terminal fragment of the bt15 gene with a NcoI site at the ATG initiation codon.

According to Brizzard and Whiteley (1988), the initiation codon of the bt14 gene is a TTG codon. Thus, a NcoI site was created in a like manner at this codon for site directed mutagenesis using a 34-mer oligonucleotide with the following sequence:

5'-CCTATTTGAAGCCATGGTAACTCCTCCTTTTATG-3'.

In this case the sequence of the intitiation codon was converted from ATATTGA to ACCATGG. This yielded the plasmid pHW44 carrying the N-terminal fragment of the bt14 gene with a NcoI site at the initiation codon.

In a second step, the genes were reconstructed by ligating the N-terminal gene fragments with a suitable C-terminal gene fragment, yielding a bt15 gene and bt14 gene with a NcoI site at the ATG initiation codon.

To express the bt14 and bt15 genes encoding the protoxin in E. coli, the following constructs were made: pOH50 containing the bt15 gene under the control of the lac promoter; and pHW67 containing the bt14 gene under the control of the tac promoter. Induction of a culture of the E. coli strain WK6 carrying the respective plasmids with IPTG yielded an overproduced protein (Fuller, 1982).

The active toxic fragments of the Bt15 and Bt14 protoxins comprise 63 and 60 kDa trypsin digest products respectively. Instead of expressing the whole bt15 or bt14 gene, it is also possible to express a toxin-encoding gene fragment or derivative thereof in plants. To this end, truncated bt14 and bt15 gene fragments were constructed. In order to be able to select transgenic plants producing the ICP gene products, hybrid genes of the truncated gene fragments were also made with the neo gene encoding a selectable marker as described in EP 0193259.

By comparison of the nucleotide sequence of the bt4, bt14 and bt15 genes, respectively, with the bt2 and bt18 genes, respectively, the BclI site could be identified as a suitable site localized downstream of the coding sequence encoding the toxin gene fragment. To construct a truncated gene fragment and a hybrid gene of the truncated gene fragment with the neo gene, the filled BclI site was ligated to the filled EcoRI site of pLKM91 (Höfte et al., 1986) and the filled HindIII site of pLK94 respectively (Botterman and Zabeau, 1987). pLKM91 carries a 5' truncated neo gene fragment which codes for an enzymatically active C-terminal gene fragment of the neo gene, and pLK94 contains translation stop codons in three reading frames. This yielded the following plasmids which are then used to transform E. coli to express the ICP

genes: pHW71 carrying a truncated bt14-neo hybrid gene; pHW72 carrying a truncated bt14 gene; pVE34 carrying a truncated bt15-neo hybrid gene; and pVE35 carrying a truncated bt15 gene.

In a similar way as described for the bt14 and bt15 genes, gene cassettes are constructed for the bt3 and bt4 genes which are then expressed in E.coli.

EXAMPLE 3: Purification of recombinant ICPs

The ICPs expressed in E. coli in Example 2 are purified by the method (described for recombinant Bt2 protoxin) by Höfte et al. (1986).

EXAMPLE 4: Purification of toxins

Solubilized protoxins of Bt2, Bt3, Bt73, Bt4, Bt14, Bt15, Bt18, Bt13, Bt21 and Bt22 (in $Na_2CO_3$ 50mM, DTT 10 mM pH=10) are dialyzed against 0.5 % $(NH_4)_2CO_3$ at pH 8 and treated with trypsin (trypsin/protoxin=1/20 w/w) for 2h at 37°C. The activated toxin is chromatographically purified (Mono-Q column on FPLC) as described by Hofmann et al.(1988b).

EXAMPLE 5: Determination of the insecticidal spectrum

The ICP protoxins and toxins of Examples 3 and 4 are evaluated for their insecticidal activity. Each protoxin is dissolved in alkaline buffer containing a reducing agent ($Na_2CO_3$ 50 mM, DTT 10 mM pH=10), and each toxin is used as soluble protein directly from FPLC. Protein concentrations are determined. Subsequently, dilutions of the resulting protoxin or toxin solution are prepared in PBS buffer pH=7.4 containing 0.15 M NaCl and 0.1 % bovine serum albumin ("BSA").

The artificial medium for insect culture, described by Bell and Joachim (1976) for Manduca sexta, is poured in appropriate receptacles and allowed to solidify. Subsequently a quantity of the (pro)toxin dilutions is applied on this medium, and the water is

allowed to evaporate under a laminar flow. This results in a medium with a certain quantity (in the range of 0.1 to 10000 ng/cm2) of toxin coated on its surface. For example, for the Bt2 toxin, typical dilutions for a toxicity test on Manduca sexta are 1, 5, 25, 125 and 625 ng/cm2. First instar larvae of Manduca sexta are then applied on the coated medium, and growth and mortality are assessed after 6 days. Mortality increases with dosage. Dose response data is analysed in probit analysis (Finney, 1962), and the data are best summarized by an $LD_{50}$ value which is the amount of toxin which kills 50 % of the insects. The $LD_{50}$ for Bt2 toxin against Manduca sexta is around 20 ng/cm2.

Similar assays are carried out for other insect species using a suitable diet or by applying the ICPs on leaves for insects, for which no artificial diet is used.

EXAMPLE 6: Binding studies

Toxins

All protoxins and their toxic fragments were purified according to the methods described for the Bt2 protoxin and toxin in Höfte et al. (1986) and EP 0193259. The activated and purified toxins are further referred to as the Bt2, Bt3, Bt73, Bt4, Bt14, Bt15, Bt18, Bt13, Bt21 and Bt22 toxins.

By way of example for the Bt73 toxin, it has been shown that B. thuringiensis var. kurstaki HD73 produces a protein of 133 kDa encoded by a 6.6 kb type gene. A culture of this strain was grown as described by Mahillon and Delcour (1984). The autolysed culture was spun down (20 minutes at 4500 rpm in a HB4 rotor) and washed with a buffer containing 20 mM Tris, 100 mM NaCl and 0.05 % Triton X-100, pH 8. The final pellet was resuspended in this buffer (4 ml buffer for 100 ml culture). This solution was then layered onto a linear

Urograffin gradient (60-70%) which was centrifuged in a SW 28 rotor for 90 minutes at 18000 rpm. Crystals were collected and stored at -20° C until further use. Activation was performed according to Höfte et al. (1986). The purified toxin is further referred to as the Bt73 toxin.

Iodination of ICPs

Iodination of Bt2, Bt3, and Bt73 toxins was performed using the Chloramin-T method (Hunter and Greenwood, 1962). 1 mCi $^{125}$I-NaI and 20 to 37.5 ug Chloramin-T in NaCl/$P_i$ were added to 50 ug of purified toxin. After gentle shaking for 60 seconds, the reaction was stopped by adding 53 ug of potassium metabisulfite in $H_2O$. The whole mixture was loaded on a PD 10 Sephadex G-25M gelfiltration column to remove free iodine. A subsequent run on a Biogel P-60 column was carried out in order to increase the purity.

Alternatively, toxins were labeled using the Iodogen method. Iodogen (Pierce) was dissolved in chloroform at 0.1 mg/ml. 100 ul of this solution was pipetted into a disposable glass vessel and dried under a stream of nitrogen gas. The vessel was rinsed with Tris buffer (20 mM Tris, pH 8.65 with 0.15 M NaCl). 50 ug of toxin (in Tris buffer) was incubated with 1 mCi of $^{125}$I-NaI in the tube for 10 minutes. The reaction was then stopped by the addition of 1 M NaI ( one fourth of the sample volume). The sample was immediately loaded onto a PD10 Sephadex G-25M column and later on a Biogel P-60 column to remove free iodine and possible degradation products.

Other toxins were iodinated using one of the above mentioned procedures.

Determination of specific activity of iodinated toxin

Specific activity of iodinated Bt2, Bt3, and Bt73 toxin samples was determined using a "sandwich" ELISA

technique according to Voller, Bidwell and Barlett (1976). Primary antibody was a polyclonal antiserum raised against Bt2 toxin, and the secondary antibody was a monoclonal antibody 4D6.

The conjugate used was alkaline phosphatase coupled to anti-mouse IgG. The reaction intensity of a standard dilution series of unlabeled toxin and dilutions of the iodinated toxin sample (in $NaCl/P_i$ - 0.1 % BSA) was measured. Linear regression calculations yielded the protein content of the radioactive toxin sample. The samples with the highest specific activities were used in the binding assays. Specific activities were 59400, 33000 and 19800 Ci/mole (on reference date) for Bt73 toxin (labeled according to Iodogen procedure), Bt2 toxin (Chloramin-T method) and Bt3 toxin (Iodogen method) respectively.

Specific activities of other toxins were determined using a similar approach. Specific monoclonal and polyclonal antibodies for each of these toxins were raised and applied in ELISA.

Preparation of brush border membrane vesicles

Brush border membrane vesicles ("BBMV") from Manduca sexta, Heliothis virescens, Plutella xylostella, Phthorimaea operculella, Spodoptera exigua, Spodoptera littoralis, Plodia interpunctella, Mamestra brassicae, Pieris brassicae and Leptinotarsa decemlineata were prepared according to the method of Wolfersberger et al. (1987). This is a differential centrifugation method that makes use of the higher density of negative electrostatic charges on luminal than on basolateral membranes to separate these fractions.

Binding assay

Duplicate samples of $^{125}I$-labeled toxin, either alone or in combination with varying amounts of

unlabeled toxin, were incubated at the appropriate temperature with brush border membrane vesicles in a total volume of 100 ul of Tris buffer (Tris 10 mM, 150 mM NaCl, pH 7.4). All buffers contained 0.1 % BSA. The incubation temperature was 20 C. Ultrafiltration through Whatman GF/F glass fiber filters was used to separate bound from free toxin. Each filter was rapidly washed with 5 ml of ice-cold buffer ($NaCl/P_i$- 0.1 % BSA). The radioactivity of the filter was measured in a gammacounter (1275 Minigamma, LKB). Binding data were analyzed using the LIGAND computer program. This program calculates the bound concentration of ligand as a function of the total concentration of ligand, given the affinity (Ka or its inverse Kd = 1/Ka, the dissociation constant) and the total concentration of receptors or binding site concentration ($R_t$).

Determination of protein concentration

Protein concentrations of purified Bt2, Bt3, Bt73 and Bt15 toxins were calculated from the OD at 280 nm (measured with a Uvikon 810 P, Kontron Instruments spectrofotometer). The protein content of solutions of other toxins and of brush border membrane vesicles (BBMV) as measured according to Bradford (1976).

Binding of Bt2, Bt3 and Bt73 toxins to BBMV of Manduca sexta and Heliothis virescens: an example of 3 competitively binding Lepidopteran ICPs.

Bt2, Bt3 and Bt73 toxins are toxic to both Manduca sexta and Heliothis virescens: LC50 values for Manduca sexta are respectively 17.70, 20.20 and 9.00 ng/cm2 ; for Heliothis virescens the LC50's are 7.16, 90.00 and 1.60 ng/cm2.

Labelled toxin, either Bt3 (0.8 nM) or Bt2 (1.05 nM) or Bt73 (1.05 nM), was incubated with BBMV in a volume of 0.1 ml. BBMV protein concentrations were 100 ug/ml for M. sexta and for Bt2-H. virescens, for Bt3-H.

virescens 150 and for Bt73-H. virescens 50 ug/ml. The labelled toxin was combined with varying amounts of an unlabeled toxin (competitor). After a 30 min. incubation, bound and free toxins were separated through filtration.

Figs. 1-3 show the percentages binding of respectively labelled Bt2, Bt3 and Bt73 toxins as a function of the concentration of competitor for Manduca sexta. Figs. 4-6 show these data for Heliothis virescens. The amount bound in the absence of competitor is always taken as 100 % binding. Figs. 1-6 show the binding of $^{125}$I-labeled toxins to M. sexta (in Figs. 1, 2 and 3) and H. virescens (in Figs. 4, 5 and 6) brush border membrane vesicles. Vesicles were incubated with labeled toxin [in Figs. 1 and 4: $^{125}$I-Bt2-toxin (1.05nM); in Figs. 2 and 5: $^{125}$I-Bt3-toxin (0.8nM); in Figs. 3 and 6: $^{125}$I-Bt73-toxin (1.05nM)] in the presence of increasing concentrations of Bt2 toxin (*), Bt3 toxin (●) or Bt73 toxin (▲). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. On M. sexta vesicles, these amounts were 1820, 601 and 2383 cpm, and on H. virescens vesicles 1775, 472 and 6608 cpm for $^{125}$I-Bt2-, Bt3- and Bt73-toxin, respectively. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample.

Figure 1: shows the binding of $^{125}$I Bt2 toxin to M. sexta BBMV

Figure 2: shows the binding of $^{125}$I Bt3 toxin to M. sexta BBMV

Figure 3: shows the binding of $^{125}$I Bt73 toxin to M. sexta BBMV

Figure 4: shows the binding of $^{125}$I Bt2 toxin to H. virescens BBMV

Figure 5: shows the binding of $^{125}I$ Bt3 toxin to H.virescens BBMV

Figure 6: shows the binding of $^{125}I$ Bt73 toxin to H.virescens BBMV

The conclusions from Figures 1-6 are that Bt2 and Bt3, Bt3 and Bt73, and Bt2 and Bt73 are competitively-binding ICP's both for Manduca sexta and for Heliothis virescens. Indeed Bt3 competes for the entire population of receptor sites of Bt2 in Manduca sexta (Fig.1): the % labelled Bt2 bound in the presence of 100 nM Bt3 is equal to the % Bt2 bound with 100 nM of Bt2 itself. The opposite is not true: in the presence of 100 nM Bt2 the % of labelled Bt3 is not reduced to the same level as with 100 nM of Bt3 (Fig.2).

A similar reasoning is followed to observe competitivity of other toxin combinations : Bt3 competes for the entire population of receptor sites of Bt73 (Fig. 3) in M. sexta; the opposite is not true (Fig. 2); Bt2 and Bt73 compete for the entire population of each other's binding sites in M. sexta (Figs. 1 and 3).

In Heliothis virescens : Bt2 competes for the entire population of receptor sites of Bt3 (Fig. 5); Bt73 competes for the entire population of receptor sites of Bt3 (Fig. 5); Bt73 competes for the entire population of receptor sites of Bt2 (Fig. 4); but the opposite statements are not true (Figs. 4, 5 and 6).

The same data can be used in mathematical analysis (e.g., Scatchard analysis according to Scatchard, 1949; analysis with the LIGAND computer program according to Munson and Rodbard, 1980) to calculate the dissociation constant (Kd) of the toxin-receptor complex and the concentration of binding sites (Rt); the results of these calculations using the LIGAND computer program were the following:

Bt2-M.sexta: Kd=0.4 nM Rt=3.4 pmol/mg vesicle protein

Bt3-M. sexta: Kd=1.5 nM Rt=9.8 pmol/mg vesicle protein

Bt73-M. sexta: Kd=0.6 nM Rt=4.0 pmol/mg vesicle protein

Bt2-H. virescens: Kd=0.6 nM Rt=9.7 pmol/mg vesicle protein

Bt3-H. virescens: Kd=1.2 nM Rt=3.7 pmol/mg vesicle protein

Bt73-H. virescens: Kd=0.8 nM Rt=19.5 pmol/mg vesicle protein

These data demonstrate the high affinity receptor binding of the toxins (Kds in the range of $10^{-10}$ to $10^{-9}$ M.

Binding of Bt2 and Bt14 toxins to BBMV of P. brassicae, Plutella xylostella and Phthorimaea operculella: an example two non-competitively binding Lepidopteran ICPs

Bt2 and Bt14 toxins are toxic to P. brassicae (p.b.), P. xylostella (p.x.) and P. operculella (p.o.) as seen from the table below.

$LC_{50}$ of Toxins

| | Bt2 | Bt14 |
|---|---|---|
| P.b. | 1.3 | 2.0 |
| P.x. | 6.7 | 5.4 |
| P.o. | 4.20 | 0.8-4.0 |

$LC_{50}$ values of solubilized purified Bt2 and Bt14 toxins for P.x. are expressed as ng protein spotted per $cm^2$ of artificial diet. $LC_{50}$ values for P.b. are expressed as $ug^2$ toxin per ml solution into which leaf discs, fed to first instar Pb larvae, were dipped. For P.o., $LC_{50}$ values are expressed in ug/ml into which potato chips were dipped prior to feeding.

Labelled Bt2 toxin (1.05 nM) or Bt14 toxin (1.4 nM) was incubated with BBMV from P. brassicae (100 ug

protein/ml) in a volume of 0.1 ml in combination with varying amounts of unlabelled Bt2 or Bt14. After a 30 min. incubation period at 22°C, the bound and free toxins were separated.

Figures 7 and 8 show the binding of $^{125}$I-labeled toxins to P. brassicae brush border membrane vesicles. Vesicles were incubated with labeled toxin [in Fig. 7: $^{125}$I-Bt2-toxin (1.05nM); in Fig. 8: $^{125}$I-Bt14-toxin (1.4nM)] in the presence of increasing concentrations of Bt2 toxin (o) or Bt14 toxin (●). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample. Figure 7 shows the binding of labelled Bt2 toxin to P. brassicae BBMV, and Figure 8 shows the binding of labelled Bt14 toxin to P. brassicae BBMV.

The competition data demonstrate the presence of high affinity binding sites both for Bt2 and Bt14, as well as the almost complete absence of competition of Bt14 for the Bt2 binding sites and of Bt14 for the Bt2 binding sites. This demonstrates that Bt2 and Bt14 are non-competitively binding toxins. Hence they are useful to prevent the development of Pieris brassicae resistance against B. thuringiensis ICP's expressed in Brassica sp.

Calculated Kd and Rt values were from these experiments were:

Bt2: Kd=2.8 nM, Rt=12.9 pmol/mg vesicle protein

Bt14: Kd=8.4 nM, Rt=21.4 pmol/mg vesicle protein.

Binding of Bt2 and Bt15 toxins to BBMV of M.sexta, M.brassicae, P. xylostella and P.interpunctella : an example of two non-competitively binding Lepidopteran ICPs

Bt2 and Bt15 toxins are both toxic to M.sexta (LC50's of 20 and 111 ng/cm2, respectively). They also show activity against M. brassicae, P. xylostella and P. interpunctella.

Labelled Bt2 (1.05 nM) or Bt15 (0.7 nM) was incubated with BBMV from M.sexta (100 ug protein/ ml) in a volume of 0.1 ml in combination with varying amounts of unlabelled Bt2 or Bt15. After a 30 min. incubation period at 22°C, the bound and free toxins were separated.

Figs. 9-10 show the binding of $^{125}$I-labeled toxins to M. sexta brush border membrane vesicles. Vesicles were incubated with labeled toxin [in Fig. 9: $^{125}$I-Bt2-toxin (1.05nM); in Fig. 10: $^{125}$I-Bt15-toxin (0.7nM)] in the presence of increasing concentrations of Bt2-toxin (o) or Bt15-toxin (●). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample. Figure 9 shows the data for binding of labelled Bt2, and Figure 10 shows the binding of labelled Bt15.

The competition data demonstrate the presence of high affinity binding sites for both Bt2 and Bt15, as well as the complete absence of competition of Bt15 for the Bt2 binding sites and of Bt2 for the Bt15 binding sites. This demonstrates that Bt2 and Bt15 are non-competitively binding toxins. Hence the combination of Bt2 and Bt15 is useful to prevent the development of resistance of M.sexta against B. thuringiensis ICP's expressed in tobacco or other crops in which Manduca sp. are a pest. Calculated Kd and Rt values are:
Bt2: Kd=0.4 nM, Rt=3.4 pmol/mg vesicle protein

Bt15: Kd = 0.3 nM Kd2=2.9 nM, Rt1= 5.9 and Rt2=6.7 pmol/mg vesicle protein (2 distinct high affinity receptor sites are present).

Similar studies were performed for M. brassicae, S. littoralis and P. interpunctella. Although LD50, Kd and Rt values differed substantially, the essential observation that Bt2 and Bt15 are both toxic and are non-competitively binding toxins was confirmed in these three insect species. Thus, it is also a useful toxin combination to prevent resistance of M. brassicae to ICP's or to prevent resistance of Spodoptera species against ICP's expressed in any of the crop plants in which Spodoptera species are a pest.

Binding of Bt2 and Bt4 toxins to BBMV of M. sexta: an example of two non-competitively binding Lepidopteran ICPs

Both Bt2 and Bt4 toxins are toxic to Manduca sexta. LD50 values are 20 and 5.4. ng/cm2, respectively. No mutual competition of Bt2 for binding of labelled Bt4 and of Bt4 for binding of labelled Bt2 was observed, demonstrating that Bt2 and Bt4 are non-competitively binding toxins.

Binding of Bt15 and Bt18 toxins to BBMV of S. littoralis: an example of two non-competitively binding Lepidopteran ICPs

Both Bt15 and Bt18 toxins are toxic to S. littoralis. LD 50 values are 93 and 88 ng toxin/$cm^2$, respectively. Labelled Bt15 (0.7 nM) or Bt18 (0.9 nM) was incubated with 100 ug of vesicle protein from S. littoralis in combination with varying amounts of unlabelled Bt15 or Bt18 toxin. After a 45 - min. incubation period, bound and free toxins were separated. Binding data demonstrate high affinity binding for both Bt15 and Bt18 to S. littoralis BBMV. As seen from Figures 11 and 12, the entire population

of receptor sites of Bt15 was not saturable with Bt18, nor was the entire population of receptor sites of Bt18 saturable with Bt15.

Binding of Bt13 and Bt22 toxins to BBMV of L. decemlineata : an example of two non-competitively binding Coleopteran ICPs.

Both Bt13 and Bt22 toxins are toxic to L. decemlineata. LD 50 values are 0.8 and 1.1 ug toxin/ml respectively. Labelled Bt13 (1 nM) or Bt22 (0.7 nM) was incubated with 100 ug of vesicle protein/ml from S. littoralis in combination with varying amounts of unlabelled Bt13 or Bt22 toxin. After a 45 min. incubation period, bound and free toxins were separated. Binding data demonstrate high affinity binding for both Bt13 and Bt22 to S. littoralis BBMV. The entire population of receptor sites of Bt13 was not saturable with Bt22. Nor was the entire population of receptor sites of Bt22 saturable with Bt13.

Binding of Bt2 and Bt18 toxins to BBMV of M. sexta: an example of two non-competitively binding Lepidopteran ICPs.

Both Bt2 and Bt18 toxins are toxic to M. sexta, and LD 50 values are 20 to 73 ng toxin/$cm^2$ respectively. Labelled Bt2 (1.05nM) or Bt18 (0.7nM) was incubated with 100 ug/ml of vesicle protein from M. sexta in combination with varying amounts of unlabelled Bt2 or Bt18 toxin. After a 45 min. incubation period, bound and free toxins were separated. Binding data (Figs. 11-12) demonstrate high affinity binding for both Bt2 and Bt18 to M. sexta BBMV. The entire population of receptor sites of Bt2 was not saturable with Bt18. Nor was the entire population of receptor sites of Bt18 saturable with Bt2. Calculated Kd and Rt values are:

Bt2: Kd= 0.4 nM, Rt= 3.4 pmol/mg vesicle protein.

Bt18: Kd1= 0.04 nM, Rt1= 2.2 pmoles/mg vesicle protein and Kd2= 168nM Rt2= 194 pmoles/mg vesicle protein (2 distinct receptor sites for Bt18 are present).

A list of non-competitively binding anti-Lepidopteran ICP combinations and anti-Coleopteran ICP combinations is given below, together with their common target insect species in which non-competitivity has been demonstrated:

Bt2-Bt15 (Manduca sexta, Plutella xylostella, Pieris brassicae, Mamestra brassicae, Plodia interpunctella)

Bt2-Bt18 (Manduca sexta, Spodoptera littoralis)

Bt2-Bt14 (Pieris brassicae, Plutella xylostella, Phthorimaea operculella)

Bt2-Bt4 (Manduca sexta)

Bt15-Bt18 (Manduca sexta, Spodoptera littoralis)

Bt14-Bt15 (Pieris brassicae)

Bt15-Bt4 (Manduca sexta, Spodoptera exigua)

Bt18-Bt4 (Manduca sexta, Spodoptera littoralis)

Bt18-Bt14 (Pieris brassicae)

Bt18-Bt4 (Manduca sexta)

Bt13-Bt21 (Leptinotarsa decemlineata)

Bt13-Bt22 (Leptinotarsa decemlineata)

Bt21-Bt22 (Leptinotarsa decemlineata)

Of course, this list of specific non-competitively binding ICP combinations for specific target insect pests is not exhaustive, and it is believed that other such ICP combinations, including combinations for yet-to-be discovered ICPs, will be found using a similar approach for any target insect species. Likewise, the foregoing list of target insect pests also is not exhaustive, and it is believed that other target insects pests (as well as the plants that are to be transformed to prevent their attack by such pests), against which the specific combinations of ICPs can be

used (e.g., the combination of the Bt2 and Bt14 ICPs in Brassica to prevent resistance of Pieris brassicae against the ICPs expressed in the plant), will be found using a similar approach.

EXAMPLE 7: Selection for resistance of Manduca sexta (tobacco hornworm)

A selection experiment involves exposing a large number of larvae to a concentration of a toxin in a diet killing (e.g., 50-90 %) of the larvae. The surviving larvae are again exposed to toxin concentrations killing a similar proportion of the larvae, and this process is continued for several generations. The sensitivity of the larvae to the toxin is investigated after each four generations of selection.

Selections for 20 generations of M. sexta were performed with Bt2 toxin alone, with Bt18 toxin alone and with a 1/4 (by weight) Bt2/Bt18 mixture. LC50 values of the reference strain for Bt2, Bt18 and the 1/4 Bt2/Bt18 mixture respectively were the following : 20 ng/cm2, 73 ng/cm2 and 62 ng/cm2 of diet.

Selection was initiated at concentrations killing around 75 % of the larvae. After 4 generations of selection, survival increased in both the Bt2 and the Bt18 selection to around 70 %, no such increase was observed in the selection with the combination of Bt2 and Bt18. Dosages were again increased to calculated LC75 values. This was repeated every 4 generations. The selection process was thus continued to the 20th generation. Final results were the following (LC50 of the 20th generation):

- Bt2 selection: LC50 was 6400 ug/g (320 times decreased sensitivity)
- Bt18 selection: LC50 was 15100 ug/g (207 times decreased sensitivity)

- Bt2/Bt18 selection: LC50 was 181 ug/g (3 times decreased sensitivity).
Thus the decrease in sensitivity was about 100 times slower in the combined selection experiment.

Receptor binding in the three selected M. sexta strains was investigated with Bt2 and Bt18 and compared to those of the reference M. sexta strain (non-selected strain). Binding characteristics of the reference strain for the Bt2 and BT18 toxins were:
Bt2: Kd = 0.4 nM, Rt=3.4 pmol/mg vesicle protein
Bt18: Kd1=0.04 nM, Rt1=2.2 pmoles/mg vesicle protein and Kd2=168nM, Rt2=194 pmoles/mg vesicle protein (2 distinct receptor sites for Bt18 are present).

Figures 11 and 12 show the binding of $^{125}$I-labeled toxins to M. sexta brush border membrane vesicle. Vesicles were incubated with labeled toxin [in Fig. 11: $^{125}$I-Bt2-toxin (1.05nM); in Fig. 12: $^{125}$I-Bt18-toxin (0.7nM)] in the presence of increasing concentrations of Bt2-toxin (o) or Bt18-toxin (●). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. Non-specific binding was not substracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample.

The Bt2 selected strain showed no detectable high affinity binding of Bt2 whereas its Bt18 binding characteristics remained close to the reference strain. (Bt18: Kd1=0.03 nM, Rt1=2.8 pmoles/mg vesicle protein and Kd2=199nM, Rt2=109 pmoles/mg vesicle protein; 2 distinct receptor sites for Bt18 are still present).

The Bt18 selected strain lost the high affinity receptor site for Bt18. The lower affinity site for Bt18 was still present in lower concentration than in the reference strain (Kd=189 nM, Rt=43 nM). Bt2 binding site concentration increased markedly compared to the

reference strain (Kd=0.4 nM, Rt=20.8 pmoles/mg vesicle protein). This strain had a Bt2 sensitivity of $LC_{50}$=4 ng/cm$^2$. Thus, its sensitivity for Bt2 had increased as compared to the reference strain ($LC_{50}$=20 ng/cm$^2$).

The Bt2/Bt18 selected strain showed a slight but statistically non-significant decrease in Bt18 binding site concentration. (Bt2 : Kd = 0.4 nM, Rt=3.4 pmol/mg vesicle protein, Bt18 : Kd1=0.04 nM, Rt1=1.0 pmoles/mg vesicle protein and Kd2=168nM, Rt2=194 pmoles/mg vesicle protein; 2 distinct receptor sites for Bt18 are present). These data demonstrate that, in the two selection lines where resistance occurred, the mechanism was situated at the receptor level. Changes in receptor site are shown to be the most likely mechanism of resistance to B. thuringiensis ICPs.

EXAMPLE 8: Mechanism of resistance of the diamondback moth to the microbial insecticide Bacillus thuringiensis.

The mechanism of development of insect resistance to ICPs has been investigated in a P. xylostella strain ("PxR"). This insect strain has developed a high level of resistance in the field against Dipel. Crystals of Dipel preparations contain a mixture of ICPs such as Bt3, Bt2 and Bt73 ICPs; in Example 6, it has been shown that these toxins are competitively binding ICPs.

Resistance to Dipel was confirmed by the toxicity data for the sensitive strain ("PxS") and for the Dipel-resistant strain ("PxR"). High levels of resistance are also observed for the Bt2 protoxin and toxin as shown in the following table :

| | $LC_{50}$ of Strains | |
|---|---|---|
| | PxS | PxR |
| Bt2 | 6.7 | > 1350 |
| Bt15 | 132.6 | 120.4 |

$LC_{50}$ data are expressed as ng protein spotted per $cm^2$ of artificial diet.

However, insect toxicity data show that there is no resistance to the Bt15 protoxin and Bt15 toxin; this ICP is not present in Dipel crystals. To investigate whether a change in toxin-membrane binding was responsible for resistance, receptor binding studies were performed with $^{125}I$-labeled Bt2 toxin and Bt15 toxin, with BBMV derived from larvae midguts of the PxR and PxS strains. The results are summarized in Table 1, below.

Table 1. Binding characteristics of Bt2 and Bt15 toxins to brush border membrane vesicles from sensitive and resistant P. xylostella.

| ICP | strain | Kd (nM) | Rt (pmol/ mg protein) |
|---|---|---|---|
| Bt2 toxin | PxS | 8.1 | 1.6 |
| | PxR | no binding detectable | |
| Bt15 toxin | PxS | 1.9 | 4.2 |
| | PxR | 3.7 | 5.8 |

Table 1 shows that there was high-affinity saturable binding of the Bt2 toxin to midgut membranes of the PxS strain, but the PxR strain showed no detectable level of Bt2 toxin binding. With the Bt15 toxin, there was significant binding to BBMW of both the PxR and PxS strains, and values are not significantly different for the two strains.

These data show that resistance in P. xylostella is due to an alteration in toxin-membrane binding. Resistance to the Bt2 toxin and the sensitivity toward the Bt15 toxin of the PxR strain is reflected by the binding characteristics shown in Table 1.

Hence, when different non-competitively binding ICPs (i.e., Bt2 and Bt15) are available with activity against the same insect species (e.g., P. xylostella),

resistance to one ICP(Bt2) does not imply resistance against other ICPs (such as Bt15). Thus, ICPs with different binding properties can be used in combination to delay development of insect resistance to ICPs.

EXAMPLE 9: Separate transfer of two ICP genes within individual transcriptional units to the genome of plant cells

Two procedures are envisaged for obtaining the combined expression of two ICP genes, such as the bt2 and bt15 genes in transgenic plants, such as tomato plants. These procedures are based on the transfer of two chimeric ICP genes, not linked within the same DNA fragment, to the genome of a plant of interest.

A first procedure is based on sequential transformation steps in which a plant, already transformed with a first chimeric ICP gene, is retransformed in order to introduce a second ICP gene. The sequential transformation makes use of two different selectable marker genes, such as the resistance genes for kanamycin ("km") and phosphinotricin acetyl transferase ("PPT"), which confers resistance to phoshinotricin. The use of both these selectable markers has been described in De Block et al. (1987).

The second procedure is based on the cotransformation of two chimeric ICP genes on different plasmids in a single step. The integration of both ICP genes can be selected by making use of the two selectable markers conferring resistance to Km and PPT, linked with the respective ICP genes.

For either procedure, a Ti-plasmid vector is used for Agrobacterium-mediated transformation of each chimeric ICP gene into plant cells.

Plasmid pGSH163, described in EP 0193259, contains the following chimeric genes between the T-DNA border

repeats: a gene fragment encoding the toxin part of the bt2 gene under the control of the TR2' promoter and the neo gene under control of the TR1' promoter. The 3' ends of the T-DNA gene 7 and octopine synthase respectively provide information for the 3' end formation of transcripts.

A chimeric bt15 gene containing a gene fragment encoding the toxin of the Bt15 ICP under the control of the TR2' promoter, was constructed in the following way (Figure 15). pOH50 consists of pUC18 with the whole bt15 gene under the control of the lac promoter. A HindIII-BglII fragment was cloned in pMa5-8 yielding pJB3. By site-directed mutagenesis, a NcoI site was created at the initiation codon to yield pVE29. A fragment containing the truncated gene fragment of the bt15 gene, with a translational stop codon, was obtained by isolation of BclI-ClaI from pOH50 and cloning in pLK91, yielding pHW38. The whole toxin gene fragment was reconstructed under the control of the tac promoter, yielding pVE35, by ligation of a ClaI-PstI fragment from pHW38, a NcoI-ClaI fragment from pVE29 and a NcoI-PstI fragment from pOH48. A truncated bt15 gene fragment with a NcoI site at the initiation codon was obtained from pVE35 as a 1980 NcoI-BamHI fragment and cloned in pGSJ141, digested with ClaI and BamHI. pGSJ141 has been described in EPA 88402115.5. Ligation of the filled ClaI site to the filled NcoI site yielded a chimeric TR2' - truncated bt15 - 3'g7 construct (pTVE47). As a selectable marker in this plasmid, the bar gene encoding phosphinothricin acetyl transferase and conferring resistance to PPT was used. A chimeric bar gene containing the bar gene under the control of the 35S promoter and followed by the 3' end of the octopine synthase was introduced in pTVE47. From pDE110, a 35S-bar-3'ocs fragment was obtained as a

StuI-HindIII fragment and was cloned in pTVE47 digested with PstI and HindIII. This yielded the plasmid pTHW88 (Figure 15) which contains the truncated bt15 gene under the control of the TR2' promoter and the bar gene under the control of the 35S promoter between the T-DNA border repeats. Plasmid pGSH163 is cointegration type Ti-plasmid vector, whereas pTHW88 is a binary type Ti-plasmid vector as described in EPA 0193259.

Both plasmids were mobilized in the A. tumefaciens strain C58C1Rif (pGV2260) according to Deblaere et al. (1988). In the sequential transformation procedure, tomato was transformed according to De Block et al. (1987) with the A. tumefaciens strain C58C1Rif carrying pGS1163 resulting from the cointegration of pGSH163 and pGV2260. Individual transformants were selected for kanamycin resistance, and regenerated plants were characterized for expression of the truncated bt2 gene according to Vaeck et al. (1987). One representative transformant was subsequently retransformed with the A. tumefaciens strain C58C1Rif (pGV2260 and pTHW88), and transformants were selected for PPT resistance. Using this cotransformation procedure, the respective Agrobacteria strains, carrying the cointegrate vector pGS1163 and the binary vector pTHW88, were used for transformation of tomato. Individual plants were selected for resistance to Km and PPT.

Schematically shown in Fig. 15 are:

a) construction of pVE29: bt15 N-terminal gene fragment with NcoI site introduced at ATG initiation codon.

b) construction of pVE35: bt15 C-terminal truncated gene fragment under control of the tac promoter.

c) construction of pTHW88: binary T-DNA vector with a chimeric bt15 gene and a chimeric bar gene within the T-DNA border repeats.

In both cases, co-expression of the two ICP genes in the individual transformants was evaluated by insect toxicity tests as described in EP 0193259 and by biochemical means. Specific RNA probes allowed the quantitive analysis of the transcript levels; monoclonal antibodies cross-reacting with the respective gene products allowed the quantitative analysis of the respective gene products in ELISA tests (EP 0193259); and specific DNA probes allowed the characterization of the genomic integrations of the bt2 and bt15 genes in the transformants. It was found that the transformed tomato plants simultaneously expressed both the bt2 gene (8.1 ng/mg) and the bt15 gene (7.6 ng/mg) as measured by ELISA, which would prevent or delay development of resistance of M. sexta to the insecticidal effects of the Bt2 and Bt15 toxins, being expressed.

These procedures also could be applied when one or both ICP genes are part of a hybrid gene. For example, the same strategy as described above could be followed with the plasmid vectors pGSH152, containing a chimeric truncated bt2-neo hybrid gene under control of the TR2' promoter, and pTHW88 in suitable Agrobacterium strains.

EXAMPLE 10: Separate transfer of two ICP genes to the nuclear genome of separate plants in independent transformation events and subsequent combination in a single plant through crossing.

Tobacco plants have been transformed with either the bt18 gene or the bt15 gene by applying the same cloning strategies as described in EP 0358557 and EP

193259, respectively. For both genes, the plants were transformed with plant expression vectors containing either the truncated bt18 or bt15 gene, which just encode the Bt18 or Bt15 toxin, respectively.

The mortality rate of Spodoptera littoralis larvae feeding on the transformed plants is significantly higher than the mortality rate of larvae fed on untransformed plants.

The bt18-transformed plant, which is homozygous for the bt18 gene, is then crossed with the bt15 - transformed plant, which is homozygous for the bt15 gene. After selfing, a plant homozygous for both genes is obtained.

The resulting tobacco plants, expressing both the bt18 and bt15 genes, delay significantly development of resistance by S. littoralis to either the Bt18 or Bt15 toxin expressed by the plants.

EXAMPLE 11: Transfer of two chimeric ICP genes linked within the same DNA to the genome of plant cells

The strategy used is based on the organization of two independent chimeric ICP genes between the T-DNA border repeats of a single vector. Binding studies indicated that the Bt2 and Bt14 toxins are two non-competitively binding ICPs with insecticidal activity towards Pieris brassicae. For expression in plants, both the bt2 and bt14 genes can be co-expressed to prevent insect resistance development. For the design of a plasmid vector with each ICP gene under the control of a separate promoter, two possibilities can be envisaged: 1) three chimeric constructs carrying the truncated bt2 and bt14 genes and a selectable marker, respectively; or 2) a hybrid of a truncated gene fragment (bt2 or bt14) and the neo gene can be used in combination with a truncated bt14 or bt2 gene.

This Example describes the construction of the vector pTHW94 for plant transformations carrying the following chimeric ICP genes between the T-DNA border repeats: a truncated bt2 gene fragment under the control of the TR2' promoter and a hybrid truncated bt14-neo gene under the control of the TR1' promoter. The 3' end of the T-DNA gene 7 and octopine synthase, respectively, provide information for proper 3' end formation. pTHW94 has been deposited at the DSM under accession no. 5514 on August 28, 1989.

Schematically shown in Fig. 16 are the:

a) construction of pHW44: bt14 N-terminal gene fragment with NcoI site introduced at ATG initiation codon.

b) construction of pHW67: reconstruction of the bt14 gene under the control of the tac promoter.

c) construction of pHW71: construction of a hybrid truncated bt14-neo gene under the control of the tac promoter.

d) construction of pTHW94: binary T-DNA vector with a chimeric bt14 gene and a chimeric bt2 gene within the T-DNA border repeats.

The pTHW94 vector is mobilized into the Agrobacterium strain C58C1Rif (pMP90) which is used to transform Brassica napus according to the procedure described by De Block et al. (1989). Transformants are selected on Km, and regenerated plants are found to express both ICP gene products in insect toxicity tests and biochemical tests.

EXAMPLE 12: Expression of two ICP genes in a hybrid construct

In order to obtain a combined and simultaneous expression of two ICP genes, truncated gene fragments encoding the toxic parts of two different ICPs can be fused in a proper reading frame and placed, as a hybrid gene, under the control of the same promoter in a chimaeric gene construct. Toxic cores from certain ICPs can be liberated from their protoxins by protease activation at the N- and/or C- terminal end. Thus, hybrid genes can be designed with one or more regions encoding protease cleavage site(s) at the fusion point(s) of two or more ICP genes.

The simultaneous co-expression of the bt2 and bt14 genes is obtained by constructing a hybrid gene composed of a truncated bt14 gene fragment fused to a truncated bt2 gene fragment. Schematically shown in Figure 17 is the construction of such a hybrid bt2-bt14 gene with a C-terminal bt2 gene fragment (bt860) encoding the toxic core of the Bt2 protoxin in frame with a C-terminal truncated bt14 gene fragment encoding the toxic core of the Bt14 protoxin. The BclI site in the bt2 gene, localized downstream of the trypsin cleavage site, is fused in frame with the NcoI site introduced at the N-terminal end of the truncated bt14 gene fragment. To this end, the plasmids pLBKm860 (EP 0193259) and pHW67 are used. pLBKm860 contains a hybrid bt2-neo gene under control of the lambda $P_L$ promoter. The bt2 gene moiety in the hybrid gene is a C-terminal truncated bt2 gene fragment, indicated as bt860 (in Fig. 17) (see also Vaeck et al, 1987). The construction of pHW67 is described in Fig. 16. pHW67 contains a C-terminal truncated bt14 gene fragment (bt14tox) with a NcoI site at the ATG initiation codon, a

translation stop codon located at the BclI site of the intact bt14 gene and a BamHI site downstream of the whole gene fragment. To fuse both gene fragments in the proper reading frame, the BclI and NcoI ends of the respective plasmids are treated with Klenow DNA polymerase and S1 nuclease as indicated in Figure 16. The resulting plasmid pJB100 contains the hybrid bt860-bt14tox gene under control of the lambda $P_L$ promoter and directs the expression in E. coli of a fusion protein with the expected mobility on SDS-PAGE.

Crude extracts of the E. coli strain show the toxicity of the fusion protein, expressed by the strain, against P. brassicae. It has also been confirmed by N-terminal amino acid sequence analyses of the fusion protein produced by the E. coli strain that the N-terminal amino acids from the Bt14 protoxin are processed upon activation. The bt2-bt14 hybrid gene product has thus two potential protease cleavage sites.

Subsequently, this hybrid gene is inserted into a vector for plant transformations and placed under control of a suitable promoter and transferred to the genome of brassica (EP 0193259) where both the bt2 and bt14 genes are expressed in insect toxicity tests.

Table 2

| Gene | Bt strain | Host range | amino acids encoded | predicted MW(kDa) of encoded aminoacids | Disclosure of nucleotide sequence |
|---|---|---|---|---|---|
| bt3 | HD-1 kurstaki | L | 1176 | 133.2 | Schnepf et al.,1985 |
| bt2 | berliner 1715 | L | 1155 | 131 | Höfte et al.,1986 |
| bt73 | HD-73 | L | 1178 | 133.3 | Adang et al, 1985 |
| bt14 | entomocidus HD-110 | L | 1207 | 138 | Brizzard and Whiteley, 1988 |
| bt15 | entomocidus HD-110 | L | 1189 | 134.8 | Fig. 14 |
| bt4 | HD-68 aizawai | L | 1165 | 132.5 | Fig. 13 |
| bt18 | darmstadiensis HD-146 | L | 1171 | 133 | EP appln. 88402241.9 |
| bt13 | Bt51,DSM4288 22/10/87 | C | 644 | 73.1 | EP appln. 88402115.5 |
| bt21 | BtPGSI208, DSM 5131, 19/1/89 | C | 651 | 74.2 | EP appln. 89400428.2 |
| bt22 | BtPGSI245, DSM 5132, 19/1/89 | C | 1138 | 129 | EP appln. 8940028.2 |
| P2 | HD-263 | L/D | 633 | 70.9 | Donovan et al, 1988 |
| Cry B2 | HD-1 | L | 633 | 70.8 | Widner and Whiteley, 1989 |

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## CLAIMS

1. A cell of a plant, characterized by: at least two B. thuringiensis ICP genes stably inserted into the genome of said plant; each of said genes encoding a different non-competitively binding ICP for an insect species; whereby at least two different ICPs can be produced by said cell which do not bind competitively to the brush border membrane of the columnar midgut epithelial cell of said insect species.

2. The cell of claim 1 wherein at least one marker gene, encoding a protein or polypeptide which renders said cell easily distinguishable from cells which do not contain said protein or polypeptide, is in the same genetic locus as at least one of said ICP genes.

3. The cell of claim 1 or 2, wherein each of said ICP genes is under the control of a separate promoter capable of directing gene expression in said cell and is provided with a separate signal for 3' end formation and within a same transcriptional unit.

4. The cell of claim 2 or 3, in which said marker DNA is under the control of a separate promoter capable of directing gene expression in said plant cell and is provided with a signal for 3' end formation within a same transcriptional unit.

5. The cell of claim 1 or 2, wherein said ICP genes are within a same transcriptional unit and under the control of a single promoter.

6. The cell of claim 5, wherein said marker gene is fused with at least one of said ICP genes and is within said same transcriptional unit and under the control of said promoter.

7. The cell of claim 5 or 6, wherein a DNA fragment, encoding a protease-sensitive or -cleavable amino acid sequence, is in said same transcriptional unit as said ICP genes and intercalated in frame between said ICP genes.

8. The cell of claim 5 or 6, wherein said ICP genes are combined in a dicistronic unit comprising an intergenic DNA sequence which allows reinitiation of translation and is in said same transcriptional unit as said ICP genes and intercalated between said ICP genes.

9. The cell of anyone of claims 1 to 8, wherein said ICP genes are genes encoding insecticidal proteins having activity against Lepidoptera species and are particularly the following genes: bt2 and/or bt73 and/or bt4 and/or bt14 and/or bt15 and/or bt18.

10. The cell of any of claims 1 to 8, wherein said ICP genes are genes encoding insecticidal proteins having activity against a Coleoptera species and are particularly the following genes: bt13 and/or bt21 and/or bt22.

11. The cell of any of claims 2 to 10 wherein said marker DNA is: an herbicide resistance gene, particularly a sfr or sfrv gene; a gene encoding a modified target enzyme for a herbicide having a lower affinity for the herbicide, particularly a modified 5-EPSP as a target for glyphosate or a modified glutamine synthetase as a target for a GS inhibitor; or an antibiotic resistance gene, particularly NPTII.

12. The cell of any of claims 3 to 6, wherein said promoter is: a constitutive promoter, particularly a 35S promoter or a 35S3 promoter; a PNOS promoter; a POCS promoter; a wound-inducible

promoter, particularly a TR1' or TR2' promoter; a promoter which directs gene expression selectively in plant tissue having photosynthetic activity, particularly a SSU promoter; or a tissue-specific promoter, particularly a tuber-specific promoter, a stem-specific promoter or a seed-specific promoter.

13. A vector suitable for transforming a cell of a plant, particularly a plant capable of being infected with Agrobacterium, comprising said ICP genes of any of claims 1 to 12.

14. A process for producing a plant having improved insect resistance and having said ICP genes of anyone of claims 1 to 12 stably integrated into the nuclear genome of their cells, characterized by the non-biological steps of transforming a cell of said plant by introducing said ICP genes into the nuclear genome of said cell and regenerating said plant and reproduction material from said cell.

15. A plant cell culture, consisting of the plant cells of anyone of claims 1 to 12.

16. A plant, consisting of the plant cells of anyone of claims 1 to 12.

17. Brassica, tomato, potato, tobacco, cotton or lettuce consisting of the plant cells of anyone of claims 1 to 12, wherein said ICP genes comprise one of the following pairs of genes: bt2 and bt18 or bt73 and bt15 or bt2 and bt18 or bt2 and bt14 or bt2 and bt4 or bt15 and bt18 or bt14 and bt15 or bt4 and bt15 or bt13 and bt21 or bt21 and bt22 or bt13 and bt22.

18. The cell of anyone of claims 1-12, made by a process as described hereinabove.

19. A method for rendering a plant resistant to an insect species by transforming the plant with said ICP genes of anyone of claims 1-12.